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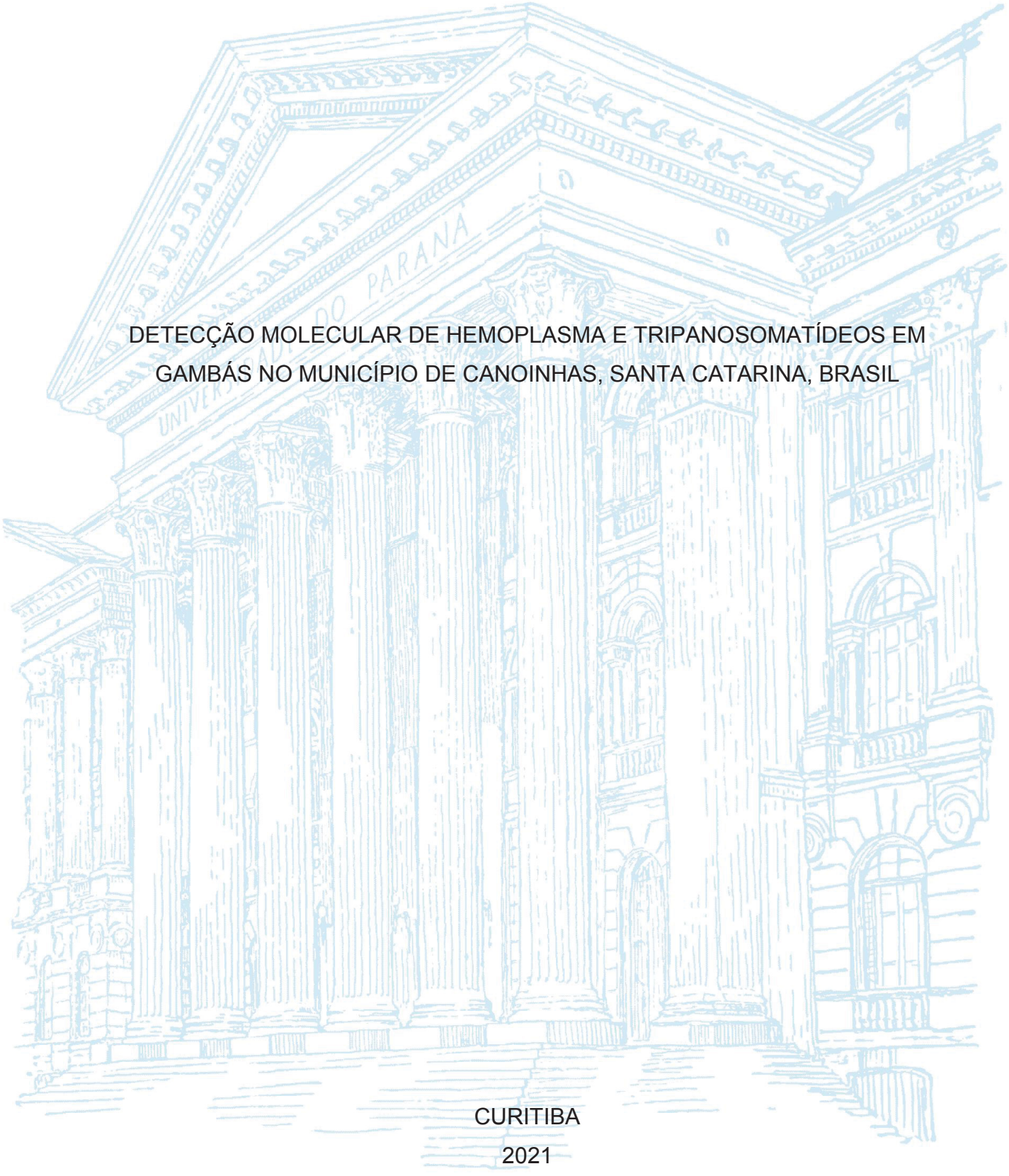

DETECÇÃO MOLECULAR DE HEMOPLASMA E TRIPANOSOMATÍDEOS EM GAMBÁS NO MUNICÍPIO DE CANOINHAS, SANTA CATARINA, BRASIL

CURITIBA

2021

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DETECÇÃO MOLECULAR DE HEMOPLASMA E TRIPANOSOMATÍDEOS EM GAMBÁS NO MUNICÍPIO DE CANOINHAS, SANTA CATARINA, BRASIL

Tese apresentada ao Programa de Pós-graduação em Ciências Veterinárias, setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de doutor em Ciências Veterinárias.

Orientador: Prof. Dr. Ivan Roque de Barros Filho

Coorientador: Prof. Dr. Rafael Felipe da Costa Vieira

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“A Medicina cura o homem, a Medicina Veterinária cura a humanidade. ”

(LOUIS PASTEUR)

RESUMO

Gambás são animais sinantrópicos e que participam do ciclo de transmissão de zoonoses. Anteriormente, duas espécies de micoplasmas hemotrópicos (hemoplasmas) foram detectados em gambás: 'Candidatus Mycoplasma haemodidelphidis' no gambá norte-americano (*Didelphis virginiana*) e um potencialmente novo Mycoplasma sp hemotrópico nos gambás-de-orelha-branca (*Didelphis albiventris)* do Brasil. A leishmaniose e a doença de Chagas são zoonoses negligenciadas na saúde pública. Nesse sentido, os objetivos deste estudo foram: i) determinar a prevalência de tripanosomatídeos e hemoplasmas em gambás de vida livre; ii) caracterizar molecularmente os tripanosomatídeos e hemoplasmas infectando gambás; iii) determinar os fatores associados à infecção de tripanosomatídeos e hemoplasmas em gambás do município de Canoinhas, Santa Catarina, sul do Brasil. Para tanto, 50 gambás-de-orelha-branca (33 capturas e 17 necropsias) foram avaliados por ensaios de reação em cadeia da polimerase (PCR) com base no gene *hsp70* e região ITS1 de tripanosomatídeos e genes 16S rRNA e 23S rRNA de pan-hemoplasma. Seis de 50 (12%; 95% CI: 5.6%-23.8%) gambás apresentaram infestação por pulgas *Ctenocephalides felis*. Vinte de 50 (40%; IC 95%: 26,41-54,82%) gambás testaram positivo para Mycoplasma sp hemotrópico. O sequenciamento e a análise filogenética dos fragmentos dos genes 16S e 23S rRNA confirmaram que os animais foram infectados por um potencial novo Mycoplasma sp hemotrópico relatado anteriormente em gambás-de-orelha-branca nos estados do Paraná e Mato Grosso do Sul, do Brasil. O nome *'Candidatus* Mycoplasma haemoalbiventris" foi proposto para este novo organismo. Não foram encontradas associações significativas entre sexo (p => 0,9999), área (p = 0,6199) ou material coletado (p = 0,8807) e positividade para tripanosomatídeos. Na análise pelo BLASTn, os fragmentos do gene *hsp70* e região ITS1, as amostras apresentaram 92,96% - 98,52% de identidade com *Trypanosoma cruzi* e 94,03% de identidade com *Leishmania infantum*, resultados estes corroborados pela filogenia. A detecção de DNA de agentes etiológicos da Doença de Chagas e Leishmaniose Visceral em gambás, considerados reservatórios silvestres, no município de Canoinhas, Santa Catarina, Sul do Brasil, são de importância epidemiológica pela ausência de casos humanos na região. A pesquisa de patógenos transmitidos por vetores e com potencial zoonótico em animais silvestres é importante para caracterizar potenciais reservatórios na epidemiologia de enfermidades de importância em Saúde Pública.

Palavras-chave: Mycoplasmas Hemotrópicos. Doença de Chagas. Leishmaniose. Marsupiais. *Didelphis albiventris*.

ABSTRACT

Opossums are synanthropic animals and can participate in the zoonosis transmission cycle. Previously, two species of hemotropic mycoplasmas (hemoplasmas) have been detected in opossums: 'Candidatus Mycoplasma haemodidelphidis' in the North American opossum (*Didelphis virginiana*) and a potentially novel hemotropic *Mycoplasma* sp. in white-eared possums (*Didelphis albiventris*) from Brazil. Leishmaniasis and Chagas disease are neglected zoonoses in public health. Accordingly, aims of this study were: i) to determine the occurrence of trypanosomatids and hemoplasmas in free-ranging opossums; ii) to molecularly characterize the trypanosomatids and hemoplasmas infecting opossums, iii) to determine factors associated with trypanosomatids and hemoplasmas infection in opossums from Canoinhas municipality, Santa Catarina State, southern Brazil. For this purpose, 50 white-eared opossums (33 captured and 17 road-killed animals) were evaluated by trypanosomatids polymerase chain reaction (PCR) assay based on *hsp70* gene and ITS1 region and were evaluated by a pan-hemoplasma PCR assay based on 16S rRNA and 23S genes. Six out of 50 (12%; 95% CI: 5.6%-23.8%) opossums were infested by *Ctenocephalides felis* fleas. Twenty out of 50 (40%; 95% CI: 26.41-54.82%) opossums tested positive for hemotropic Mycoplasma sp. by PCR. Sequencing and phylogenetic analysis of the 16S and 23S rRNA gene fragments confirmed that animals were infected by a potentially novel hemotropic *Mycoplasma* sp. previously reported in white-eared opossums in the states of Paraná and Mato Grosso do Sul, in Brazil. The name 'Candidatus Mycoplasma Haemoalbiventris' proposed this novel organism. No significant association was found between gender ($p = 0.7759$), trap area ($p = 0.0887$) or presence of fleas ($p = 0.3811$) and positivity for hemoplasmas. A total of 05/50 (10%; 95% CI: 3.33-21.81%) opossums tested positive for trypanosomatids by the PCR assay targeting the ITS1 region and *hsp70* gene. No significant associations were found between gender ($p = >0.9999$), trap area ($p = 0.6199$) or collected material ($p = 0.8807$) and positivity to trypanosomatids. In the analysis by BLASTn, the *hsp*70 gene fragments, the samples showed 92.96% - 98.52% identity with *Trypanosoma cruzi* and the ITS1 region 94.03% identity with *Leishmania infantum*, results corroborated by phylogeny. The detection of DNA of etiological agents of Chagas Disease and Visceral Leishmaniasis in opossums, considered wild reservoirs, in the municipality of Canoinhas, Santa Catarina,

southern Brazil, is of epidemiological importance due to the absence of human cases in the region. The search for vector-borne pathogens with zoonotic potential in wild animals is important to characterize potential reservoirs in the epidemiology of diseases of public health importance.

Keywords: Hemotropic Mycoplasmas. Chagas disease. Leishmaniasis. Marsupials. *Didelphis albiventris*.

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LISTA DE ABREVIATURAS

BLAST	- Basic Local Alignment Search Tool
DNA	- Ácido Desoxirribonucleico
*hsp*70	- Heat Shock Protein 70
ITS1	- Internal transcribed space 1
PCR	- Reação em cadeia da polimerase
rRNA	- Ácido ribonucleico ribossômico

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1 APRESENTAÇÃO

Didelphidae é a maior família de marsupiais das Américas e atualmente contém 19 gêneros e 95 espécies. No Brasil, 16 gêneros e 55 espécies foram descritos (ROSSI et al., 2006; NASCIMENTO; HORTA, 2014). Gambás são marsupiais pertencentes ao gênero *Didelphis*, com quatro espécies relatadas na América do Sul (*D. albiventris, D. aurita, D. imperfecta e D. marsupialis*) e uma na América do Norte (*D. virginiana*) (NASCIMENTO; HORTA, 2014).

Os didelfídeos tem alta capacidade de adaptação, memorização e resistência a endocruzamentos, o que lhes confere ampla distribuição e dispersão da espécie. Resistem a ações antrópicas no meio ambiente, adaptam-se a áreas devastadas e urbanas, buscam abrigos em residências e se alimentam de restos alimentares do homem. São considerados animais sinantrópicos e importantes modelos para estudos ecológicos e evolutivos (JANSEN, 2002; MALTA; LUPPI, 2007).

A participação dos gambás na epidemiologia de doenças é importante para a manutenção de agentes na natureza, em vetores e demais hospedeiros vertebrados, incluindo os seres humanos. Estudos conduzidos evidenciaram que os gambás podem servir como amplificadores para a transmissão horizontal de agentes patogênicos (MELO et al., 2016).

Dessa forma, devido sua ecologia e resistência a determinados patógenos, os gambás podem apresentar papel significante na epidemiologia das doenças transmitidas por vetores, emergência de patógenos zoonóticos e, desta forma, serem utilizados como sentinelas para avaliação do risco ambiental para seres humanos (DASZAK; CUNNINGHAM; HYATT, 2001).

Para tanto, 50 gambás-de-orelha-branca (33 capturados e 17 necropsiados) foram avaliados quanto à presença de DNA de Hemoplasmas e Tripanosomatídeos no município de Canoinhas Santa Catarina, Brasil.

Este estudo foi realizado em parceria com o Instituto Carlos Chagas, Fiocruz, Curitiba – PR; Universidade do Contestado, Canoinhas – SC; Universidade Estadual Paulista, Jaboticabal – SP, e Polícia Militar Ambiental, Canoinhas - SC. Aprovado pelo Comitê de Ética no Uso de Animais da Universidade do Contestado (protocolo número 06/18), conforme Anexo 1. Os procedimentos em animais e laboratoriais foram aprovados e realizados de acordo com os regulamentos do

Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, protocolo número 64418-1), conforme Anexo 2.

1.1 MICOPLASMAS HEMOTRÓPICOS

Hemoplasmas são bactérias da classe Mollicutes, ordem Mycoplasmatales, hemotrópicas, Gram negativos, sem parede celular, anteriormente classificadas como riquétsias dos gêneros *Haemobartonella* e *Eperythrozoon*. Entretanto, os resultados de sequenciamento dos genes RNA ribossômico e de ribonuclease dos genes P RNA posicionaram tais agentes dentro do gênero *Mycoplasma*. São parasitas de hemácias, podem provocar anemia hemolítica, e podem ser transmitidos por ectoparasitos (MCVEY; KENNEDY; CHENGAPPA, 2016. MESSICK; HARVEY, 2015).

Alguns membros do grupo dos micoplasmas hemotrópicos (hemoplasmas) recebem a designação de Candidatus em virtude da impossibilidade de cultivo in vitro e ausência de características taxonômicas e bioquímicas (MEGID; RIBEIRO; PAES, 2016).

Hemoplasmas foram detectados em hospedeiros vertebrados, como felinos, suínos, bovinos, camelídeos, roedores, cães e nos gambás. Neste último identificou-se a espécie ‘Candidatus Mycoplasma haemodidelphidis’ no gambá norte-americano (*Didelphis virginiana*) (MESSICK; HARVEY, 2015).

Microrganismos hemotrópicos similares aos de animais foram observados em exames citológicos de sangue humano de pacientes imunocomprometidos. Com o advento da técnica de reação em cadeia da polimerase (PCR) foram detectadas infecções de seres humanos com Mycoplasma e Bartonella, sugerindo transmissão por vetores, mordidas ou arranhaduras e manipulação de sangue animal (MESSICK; HARVEY, 2015).

O capítulo 2 desta tese apresenta uma potencial nova espécie de *Mycoplasma* sp. hemotrópico nos gambás de orelhas brancas (*Didelphis albiventris*) do Brasil. Nesse sentido, os objetivos deste capítulo foram:

a) determinar a prevalência de *Mycoplasma* spp. em gambás de vida livre,

b) caracterizar molecularmente *Mycoplasma* sp. hemotrópico infectando gambás,

c) determinar os fatores associados à infecção por hemoplasma em gambás do município de Canoinhas, estado de Santa Catarina, sul do Brasil.

Este capítulo foi publicado na revista Transboundary and Emerging Diseases com o título 'Candidatus Mycoplasma haemoalbiventris', a novel hemoplasma species in white-eared opossums (*Didelphis albiventris*) from Brazil (PONTAROLO et al., 2020).

1.2 TRIPANOSOMATÍDEOS

A ordem dos protozoários Trypanosomatidae (Kinetoplastida) inclui os gêneros *Trypanosoma* e *Leishmania* e compreende agentes etiológicos de doenças negligenciadas nos seres humanos e animais. O gênero *Trypanosoma* contém protozoários que infectam sanguessugas, insetos, peixes, anfíbios, répteis, aves e mamíferos (BARRETT et al., 2003).

Trypanosoma cruzi é o agente etiológico da Doença de Chagas, infectando pessoas, mamíferos selvagens e domésticos. A transmissão pode ocorrer por transfusão sanguínea, doação de órgãos, ingestão de alimentos ou bebidas contaminados com o protozoário, via transplacentária e por meio de vetores triatomíneos (FERNANDES et al., 1989; FRAGA et al., 2016; DROZINO et al., 2019).

Os vetores são insetos da subfamília Triatominae (Hemiptera, Reduviidae), conhecidos popularmente como barbeiro, chupão, procotó ou bicudo. Tanto os machos quanto as fêmeas, em todas as fases de seu desenvolvimento, são hematófagos. O vetor (triatomíneo), ao se alimentar em mamíferos com elevadas parasitemias de *T. cruzi,* pode se infectar e, transmitir a outros mamíferos, inclusive o homem. Quatis, gambás, tatus e morcegos podem atuar como reservatórios (BRASIL, 2019).

O vetor pica e defeca ao mesmo tempo, contendo a forma tripomastigota nas fezes que passam à ferida na pele ao coçar ou esfregar. Os tripomastigotas invadem as células e se transformam em amastigotas. Os amastigotas multiplicam-se dentro das células assexuadamente, transformam-se em tripomastigotas e destroem a célula saindo para o sangue. Tripanomastigotas sanguíneos são ingeridos por novo inseto durante a picada. Transformam-se em epimastigotas no intestino do inseto, multiplicam-se e transforma-se em tripomastigotas metacíclicas (BRASIL, 2019).

Leishmaniose é o termo utilizado para definir doenças causadas por espécies de protozoários do gênero *Leishmania* em animais e seres humanos, apresentando variações de hospedeiros reservatórios em diferentes regiões geográficas, incluindo animais domésticos e selvagens. No Novo Mundo são transmitidas por vetores do gênero *Lutzomyia* e no Velho Mundo pelo gênero *Phlebotomus* (SILVA et. al., 2016).

A leishmaniose é uma zoonose que afeta animais selvagens (reservatórios), animais domésticos e o homem. No Brasil a leishmaniose tegumentar (leishmaniose cutânea, muco-cutânea e cutânea difusa) tem como agentes etiológicos a *L. guyanensis*, *L. brasiliensis* e *L. tropica*; por outro lado, a Leishmaniose visceral possui como agente etiológico a *L. infantum* (FOGANHOLI; ZAPPA, 2011; BRASIL, 2019).

O protozoário completa o seu ciclo biológico em dois hospedeiros. As fêmeas dos flebotomíneos alimentam-se no hospedeiro vertebrado infectado e ingerem macrófagos contendo as amastigotas que se transformam na forma promastigota no inseto. As formas promastigotas são transmitidas ao novo hospedeiro vertebrado durante a alimentação do inseto. Os cães são considerados as principais fontes de infecção em área urbana. No ambiente silvestre, os reservatórios são marsupiais incluindo *Didelphis albiventris* (BRASIL, 2019).

A leishmaniose e a doença de Chagas são zoonoses negligenciadas na saúde pública. Gambás do gênero *Didelphis* são considerados animais sinantrópicos devido ao contato próximo com seres humanos. Nesse sentido, o capítulo 3 desta tese, teve como objetivos:

a) determinar a prevalência de tripanosomatídeos em gambás de vida livre;

b) caracterizar molecularmente os tripanosomatídeos infectando gambás;

c) determinar os fatores associados à infecção por tripanosomatídeos em gambás do município de Canoinhas, Santa Catarina, sul do Brasil.

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2 CANDIDATUS MYCOPLASMA HAEMOALBIVENTRIS', A NOVEL HEMOPLASMA SPECIES IN WHITE-EARED OPOSSUMS (*Didelphis albiventris*) FROM BRAZIL

Abstract

Opossums of the genus Didelphis are considered synanthropic animals due to their close contact with human beings. Previously, two species of hemotropic mycoplasmas (hemoplasmas) have been detected in opossums: '*Candidatus Mycoplasma haemodidelphidis'* in the North American opossum (*Didelphis virginiana*) and a potentially novel hemotropic *Mycoplasma* sp. in the white-eared opossums (*Didelphis albiventris*) from Brazil. Accordingly, the aims of this study were: i) to determine the prevalence of hemotropic *Mycoplasma* spp. in free-ranging opossums, ii) to characterize molecularly the hemotropic *Mycoplasma* sp. infecting opossums, iii) to determine factors associated with hemoplasma infection in opossums from Canoinhas municipality, Santa Catarina State, southern Brazil. For this purpose, 50 white-eared opossums (33 captured and 17 road-killed animals) were evaluated by a pan-hemoplasma PCR assay based on 16S rRNA. Six out of 50 (12%; 95% CI: 5.6%-23.8%) opossums were infested by *Ctenocephalides felis* fleas. Twenty out of 50 (40%; 95% CI: 26.41-54.82%) opossums tested positive for hemotropic Mycoplasma sp. by PCR. Sequencing and phylogenetic analysis of the 16S and 23S rRNA gene fragments confirmed that animals were infected by a potentially novel hemotropic *Mycoplasma* sp. previously reported in white-eared opossums from Brazil. No significant association was found between gender ($p = 0.7759$), trap area ($p = 0.0887$) or presence of fleas ($p = 0.3811$) and positivity for hemoplasmas. The potentially novel hemoplasma species seems to be highly prevalent in white-eared opossums from the states of Paraná, Santa Catarina and Mato Grosso do Sul. Based on the phylogenetic analyses of the 16S rRNA and 23S rRNA genes along with epidemiological data, the name '' is proposed for this novel organism.

Keywords: 'Candidatus Mycoplasma haemodidelphidis', Didelphis sp. hemotropic mycoplasmas, marsupials.

2.1 INTRODUTION

The family Didelphidae is the largest family of marsupials in the Americas and currently contains 19 genera and 95 species. In Brazil, 16 genera and 55 species have been described (ROSSI et al., 2006; NASCIMENTO; HORTA, 2014). Opossums are marsupials belonging to the genus Didelphis, with four species reported in South America (*D. albiventris, D. aurita, D. imperfecta and D. marsupialis*) and one in North America (*D. virginiana*) (NASCIMENTO; HORTA, 2014). Opossums are recognized as synanthropic animals, due to their ability to adapt to urban and devasted areas (MALTA; LUPPI, 2007).

Hemotropic mycoplasmas (hemoplasmas) are Gram-negative bacteria that attach to the erythrocytes' surface from vertebrate hosts and may cause hemolytic anemia (MESSICK et al., 2004). The first report of hemoplasma infection among marsupials was performed in the North American opossum (*D. virginiana*) from the USA. Based on light and electron microscopy features and 16S rRNA gene sequence analysis, the hemoplasma species was named 'Candidatus Mycoplasma haemodidelphis' (MESSICK et al., 2000; 2002). Besides, a potential novel hemoplasma species has been detected infecting white-eared opossums (*D. albiventris*) from the southern (MASSINI et al., 2019) and Central-Western (GONÇALVES et al., 2020) regions Brazil, based on 16S rRNA gene sequence analysis. Therefore, the aims of this study were: i) to determine the prevalence of hemotropic mycoplasmas in free-ranging opossums; ii) to molecularly characterize the hemoplasmas infecting opossums, iii) to determine factors associated with hemoplasma infection in opossums from Canoinhas municipality, Santa Catarina State, southern Brazil.

2.2 MATERIAL AND METHODS

2.2.1 Study area

The study was carried out in Canoinhas municipality (50° 23' 25" W, 26° 10' 38" S). Canoinhas is located in the northern region of Santa Catarina State, southern Brazil, which is characterized by semideciduous Atlantic Forest fragments and has a temperate climate with an annual average temperature of 17°C.

2.2.2 Ethical approval

This study was approved by the Ethics Committee on Animal Use of the Universidade do Contestado (protocol number 06/18). Animal and laboratory procedures were approved and performed under regulations of the Chico Mendes Institute for Biodiversity Conservation (ICMBio, protocol number 64418-1).

2.2.3 Sampling

Between October 2018 and May 2019, 50 white-eared opossums (29 were females and 21 males) (LEMOS; CERQUEIRA, 2002) were captured in rural and urban areas of Canoinhas municipality, using Tomahawk traps (Figure 1) baited with fruit (HUMBERG et al., 2012; PEREIRA et al., 2018). Sampling was performed by spontaneous demand of the Environmental Military Police of Canoinhas municipality and based on the report of the occurrence of opossums in human dwellings. An effort of 589 trap-night (number of trap * number of days) yielded 33 captures, with a success of 6.92% in rural area (20/289) and 4.33% in urban area (13/300) (MONTEIRO-FILHO; ABE, 1999; VOLOKHOV et al., 2017).

FIGURE 1- CAPTURE THE OPOSSUM IN CANOINHAS MUNICIPALITY USING TOMAHAWK TRAPS.

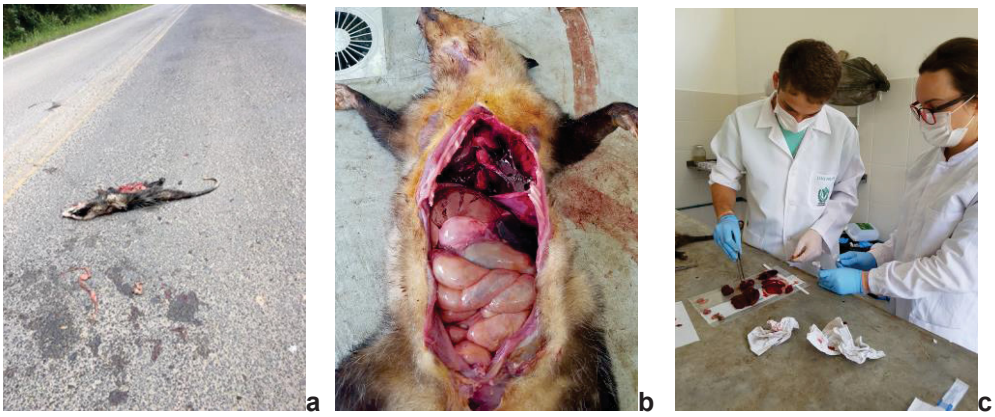
a **b** **c**

SOURCE: The author (2020).

SUBTITLE: **a**) Capture the opossum using Tomahawk traps, **b**) rural area, **c**) urban area.

Additionally, 17 road-killed (Figure 2) opossums were also evaluated. Geographical coordinates from the location of the sampled opossums were recorded (GPSMAP® 64 series, Garmin® International Inc., KS, USA).

FIGURE 2 - ROAD-KILLED OPOSSUMS EVALUATED IN CANOINHAS MUNICIPALITY.



SOURCE: The author (2020).
SUBTITLE: **a)** road-killed opossums, **b**) necropsy, **c)** fragments of spleen and liver tissues were collected.

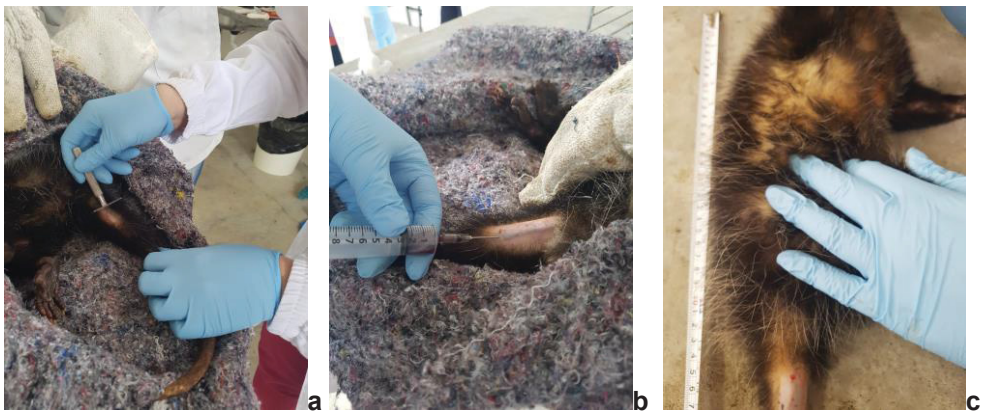
After chemical restraint (NASCIMENTO; HORTA, 2014), opossums were identified (Figure 3) with ear tagging (CÁCERES; GRAIPEL; CHEREM, 2012) and inspected for ticks and fleas.

FIGURE 3- CHEMICAL RESTRAINT, IDENTIFICATION WITH EAR TAGGING AND INSPECTION FOR TICKS AND FLEAS IN OPOSSUMS CAPTURED IN CANOINHAS MUNICIPALITY.

SOURCE: The author (2020).
SUBTITLE: **a)** weighing and identification, **b**) chemical restraint with xylazine 5.0 mg/kg and ketamine 20 mg/kg, **c**) ear tagging.

Subsequently, EDTA blood samples (Figure 4) were taken and stored at –20 °C until PCR analysis. After the procedures, opossums were monitored and later released at the same place where they were captured. Fragments of spleen (n = 15) and liver (n = 2) tissues were collected from road-killed opossums and stored –20 °C until molecular analysis. The identification of fleas followed Linardi and Guimarães (2000).

FIGURE 4 - BLOOD SAMPLES WERE TAKEN AND INSPECTION FOR TICKS AND FLEAS IN OPOSSUMS CAPTURED IN CANOINHAS MUNICIPALITY.



SOURCE: The author (2020).
SUBTITLE: **a**) trichotomy, **b**) caudal venipuncture, **c**) inspection for ticks and fleas.

2.2.4 DNA extraction

DNA was extracted from all blood and tissue samples using a commercially available kit (DNeasy® Blood & Tissue, Qiagen, Germany), according to the manufacturer's guidelines. In order to monitor for cross-contamination, ultrapure water was used in parallel as a negative control. DNA was evaluated by fluorimetry for concentration and purity using a Qubit® dsDNA HS Assay (Qubit® 2.0 Fluorometer, Invitrogen, CA, USA).

2.2.5 PCR assays

A PCR for the mammal endogenous gene glyceraldehyde-3-phosphate dehydrogenase (gapdh) (BIRKENHEUER et al., 2003) was performed in all samples, to ensure successful DNA extraction. Subsequently, DNA samples were tested by a genus-specific PCR assay targeting a fragment (900 bp) of the 16S rRNA gene of

hemoplasmas (HOELZLE et al., 2011; MACHADO et al., 2017). The amplified PCR products were subjected to gel electrophoresis in SYBR safe stained-agarose gels (1.5%) (SYBR® Safe DNA Gel Stain, Invitrogen, CA, USA) for 1 h at 100 V, and visualized.

Opossum DNA samples that showed to be positive in the screening PCR assay based on 16S rRNA were submitted to a genus-specific PCR assay targeting a fragment (800 pb) of the 23S rRNA gene of hemoplasmas (MONGRUEL et al., 2020). Nuclease-free water and *Mycoplasma haemocanis* DNA obtained from a naturally infected dog (*Canis familiaris*) blood sample were used as negative and positive controls, respectively, in both PCR assays.

2.2.6 Sequencing

Amplicons (~ 800 bp) of the 16S rRNA and 23S rRNA genes obtained from hemoplasma positive samples were purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, EUA), evaluated by spectrophotometry for concentration and purity (NanodropTM One Spectrophotometer, Thermo Fisher Scientific, Wilmington, MA, USA), and sequenced by the Sanger method. The assembled consensus sequences were generated using Geneious Prime v. 2019.2.3 and subjected to BLASTn analysis (Altschul et al., 1990) for determining the identity with sequences previously deposited in the GenBank®. Hemoplasma-16S rRNA and 23S rRNA nucleotide sequences obtained in the present study were deposited in the GenBank database (accession numbers: MT170012-MT170016 and MN442081-MN442085).

2.2.7 Phylogenetic analysis

The consensus sequences of hemoplasma-16S rRNA and 23S rRNA gene fragments were subjected to multiple alignment with sequences retrieved from GenBank® using MAFFT available on the GUIDANCE2 server (SELA et al., 2015) for each gene. The best-fit model of nucleotide substitution was determined using jModeltest v.2.1.10 (DARRIBA et al., 2012) and was set as GTR+I+G based on the Akaike Information Criterion (AIC). Each Bayesian reconstructions were realized in the Beast 1.10.4 (Drummond et al., 2012) with three independent runs of 100 million

MCMC steps sampled at every 10,000 trees, 10% of burn-in. The phylogenetic tree was visualized with FigTree software version 1.4.4 (RAMBAUT, 2016) and the final layout was done with Inkscape version 0.92.2.

2.2.8 Genotype analysis of hemoplasmas

Sequences of the hemoplasma-16S and 23S rRNA genes from five samples were analyzed to determine the number of genotypes using the DNASP software version 5.10.1 (LIBRADO; ROZAS, 2009). The genetic relationship among hemoplasmas genotypes detected herein and those previously detected in marsupials and other mammals retrieved from GenBank® were investigated by constructing a Neighbor-Net network, using the pairwise genetic distances with SplitsTree v4.14.6 (HUSON; BRYANT, 2006). The number of genotypes [h] and the average number of nucleotide differences [K]) between the novel hemoplasma species detected in the present study and '*Candidatus Mycoplasma haemodidelphidis*' were inferred using DnaSP v5 software version 5.10.01 (LIBRADO; ROZAS, 2009).

2.2.9 Statistical analyses

The Fisher's exact test was used to determine the difference between whether individual factors (gender, trap area and presence of fleas) were associated with infection by hemoplasmas. Odds ratio (OR), 95% confidence interval and p values were calculated for each variable. Results were considered significantly different when $P < 0.05$. Data were compiled and analyzed in program GraphPad Prism (version 6).

2.3 RESULTS

Six out of 50 (12%; 95% CI: 5.6%-23.8%) opossums were infested by Ctenocephalides felis fleas. All animals were not infested by ticks at the time of sampling. All samples consistently amplified the mammal endogenous gapdh gene. A total of 20/50 (40%; 95% CI: 26.41-54.82%) opossums tested positive for hemotropic Mycoplasma sp. by the PCR assay targeting the 16S rRNA gene. No

significant associations were found between gender (p = 0.7759), trap area (p = 0.0887) or presence of fleas (p = 0.3811) and positivity to hemoplasmas (Table 1).

TABLE 1 - PREVALENCE OF HEMOTROPIC MYCOPLASMAS IN OPOSSUMS WITHIN EACH VARIABLE STUDIED, SANTA CATARINA STATE, SOUTHERN BRAZIL.

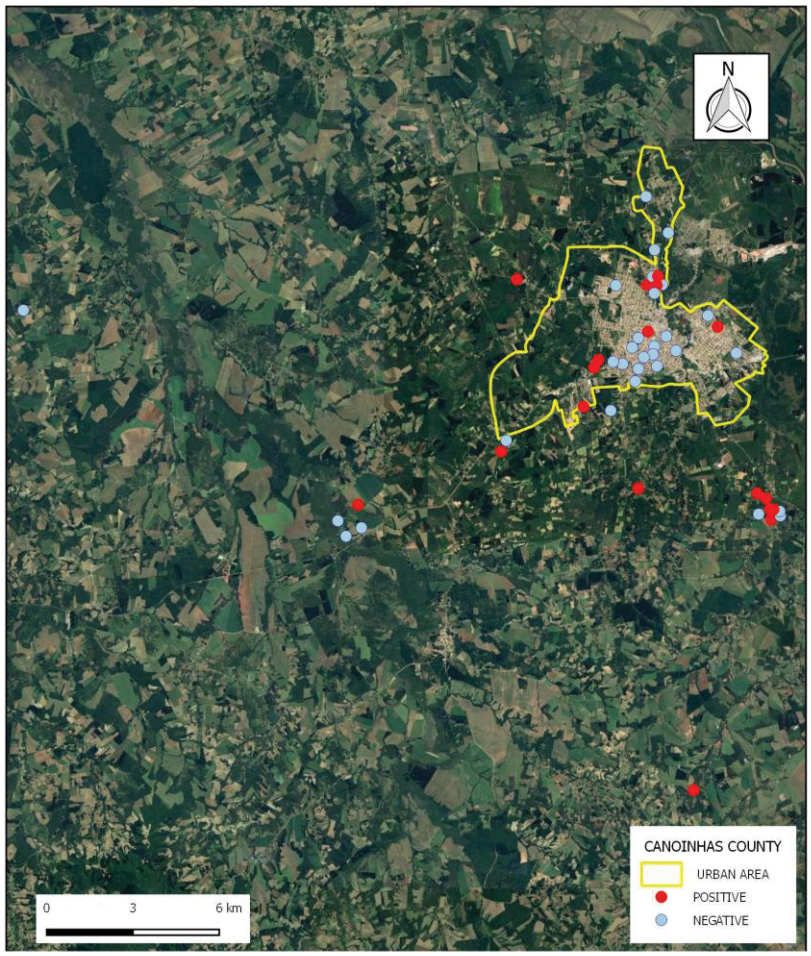
Variable		**Hemotropic mycoplasmas**		
		+/n	**OR (95% CI)**	**p-value**
Gender	Male	9/21 (42.86)	0.8148(0.2594-2.560)	0.7759
	Female	11/29 (37.93)		
Trap Area	Rural	11/20 (55.00)	0.3506 (0.1080-1.138)	0.0887
	Urban	9/30 (30.00)		
Presence of Fleas	Yes	1/6 (16.67)	0.2632(0.0283-2.445)	0.3811
	No	19/44 (43.18)		

+, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval.
SOURCE: The author (2020).

Sequencing of the 16S rRNA gene fragments from all five hemoplasma-positive samples showed 100% identity with multiple hemotropic mycoplasmas 16S rRNA gene sequences detected in white-eared opossums (*D. albiventris*) from Brazil (GenBank® accession no. MH158514, MH158515, MN423256, MN423258-MN423260). Additionally, all 16S rRNA gene sequences showed 98.88% identity with 'Ca. M. haemodidelphis' (GenBank® accession no. AF178676) identified in the North American opossum from USA, 97.27-97.52% identity with Mycoplasma sp. (GenBank® accession no. KC920439, KC920441, KC920442, KC920448) identified in raccoons (*Procyon lotor*) from the USA, and 93.42% identity with *Mycoplasma* sp. (GenBank® accession no. MH734379) detected in non-human primates (*Alouatta* sp.) from Brazil.

The geographic distribution of hemoplasmas-PCR-positive and negative opossums is shown on Figure 5.

FIGURE 5- GEOGRAPHIC POSITION OF CAPTURED POSITIVE AND NEGATIVE OPOSSUMS FOR HEMOPLASMAS.

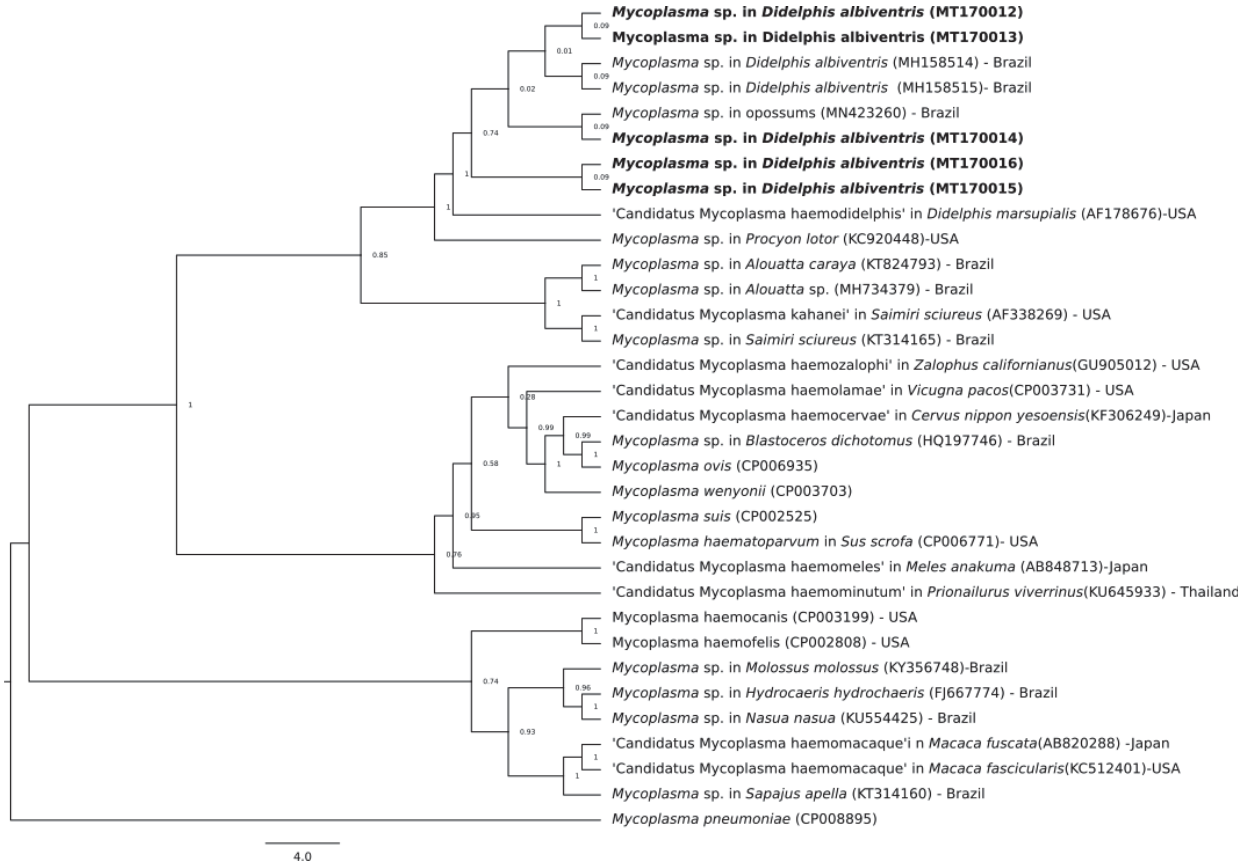


SOURCE: The author (2020).

Sequencing of the 23S rRNA gene fragments from all five hemoplasma-positive samples showed 88.15% identity with 'Candidatus Mycoplasma haemolamae' (GenBank® accession no. NR_076983) detected in an alpaca (*Vicugna pacos*) from USA, and 87.70% identity with Mycoplasma suis (NR_103970).

Phylogenetic analysis based on 16S rRNA gene fragment confirmed the close relationship of the white-eared opossum hemotropic mycoplasma genotype with 'Ca. M. haemodidelphis' identified in North American opossum. Furthermore, the genotype detected in the present study and the 'Ca. M. haemodidelphis' identified in North American opossum formed a strongly supported branch with the hemoplasma identified in a raccoon (*P. lotor*) from the USA (Figure 6).

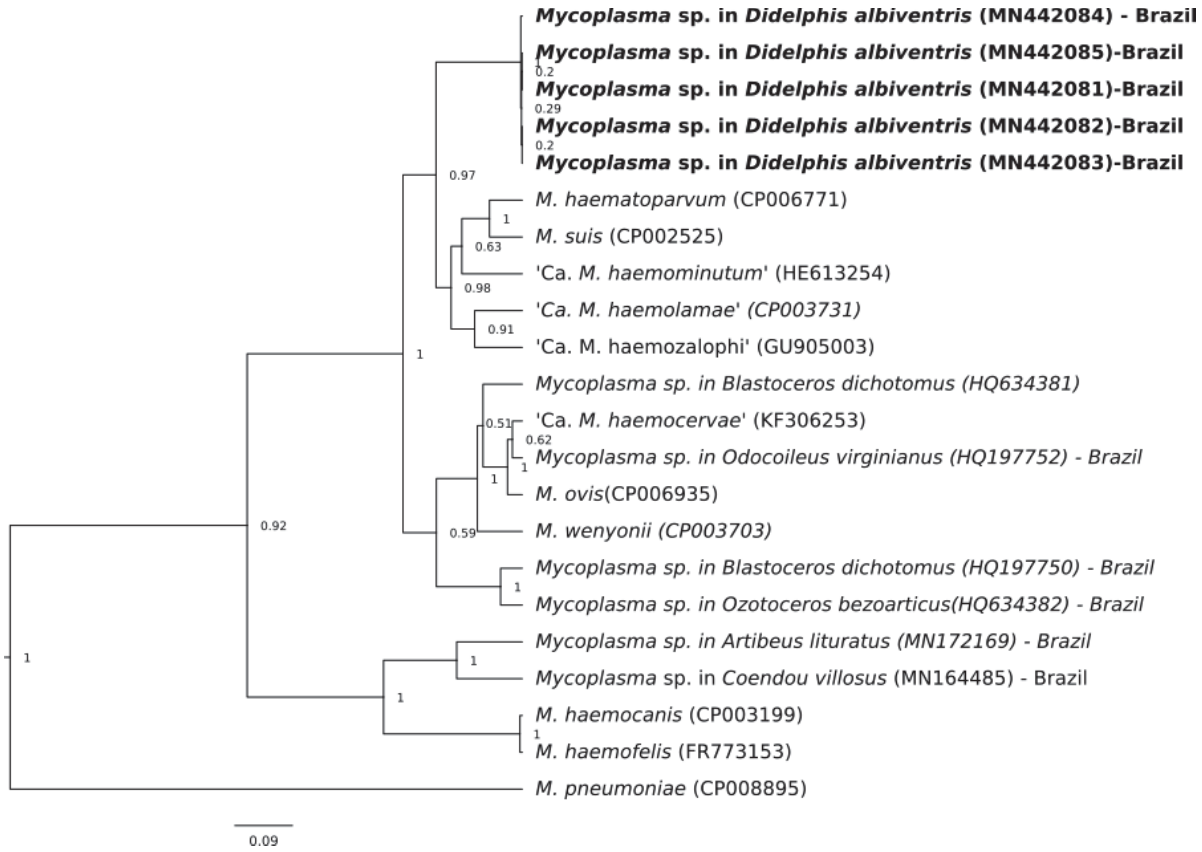
FIGURE 6 - PHYLOGENETIC TREE BASED ON PARTIAL SEQUENCES OF THE 16S RRNA GENE (850 BP), SHOWING THE RELATIONSHIP BETWEEN THE HEMOTROPIC MYCOPLASMA SP. DETECTED IN THE WHITE-EARED OPOSSUMS (*Didelphis albiventris*) FROM THIS STUDY AND OTHER HEMOPLASMAS. MYCOPLASMA PNEUMONIAE WAS USED AS OUTGROUP. THE GENBANK ACCESSION NUMBER IS IN PARENTHESES AFTER THE SPECIES NAME AND ORIGIN OF EACH AGENT. BAYESIAN INFERENCES WERE CARRIED OUT APPLYING THE GTR+I+G MODEL AND 1000 BOOTSTRAP REPLICATES FOR ALL ANALYSES.



SOURCE: The author (2020).

Phylogenetic analysis based on 23S rRNA gene fragment of the white-eared opossum hemotropic mycoplasma detected herein formed a strong supported branch and was positioned within the *M. suis* group (Figure 7).

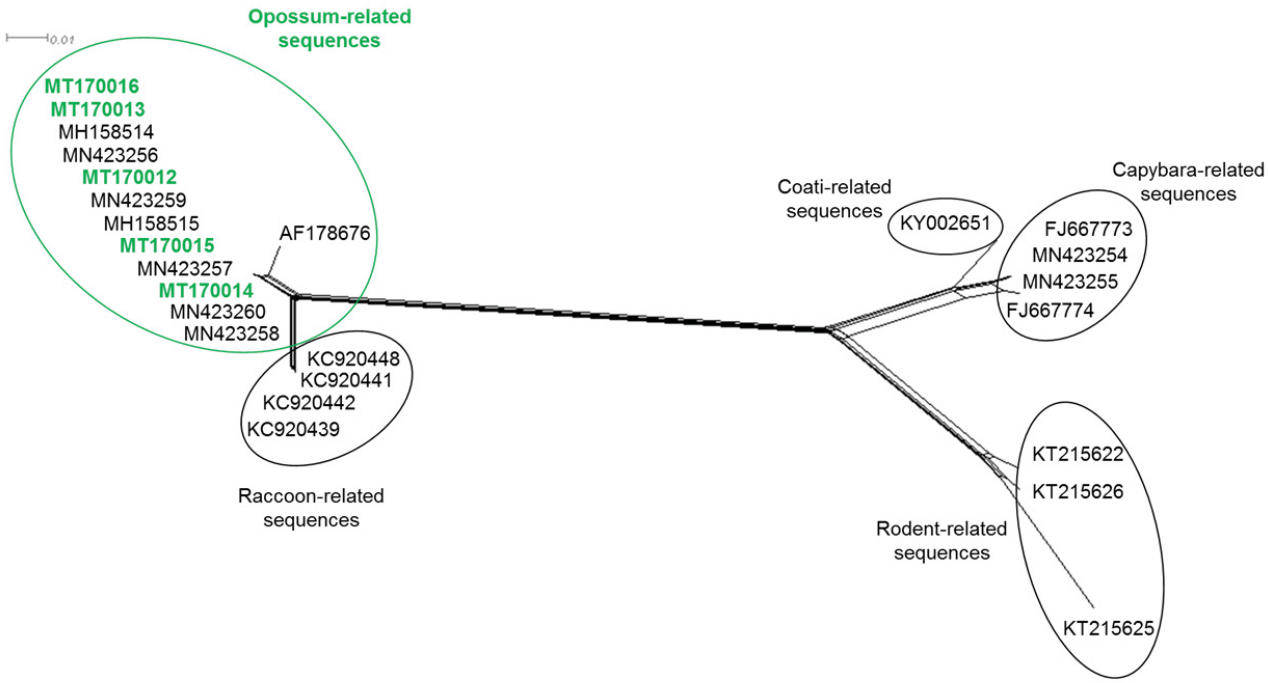
FIGURE 7 - PHYLOGENETIC TREE BASED ON PARTIAL SEQUENCES OF THE 23S rRNA GENE (785 BP), SHOWING THE RELATIONSHIP BETWEEN THE HEMOTROPIC MYCOPLASMA SP. DETECTED IN THE WHITE-EARED OPOSSUMS (*Didelphis albiventris*) FROM THIS STUDY AND OTHER HEMOPLASMAS. MYCOPLASMA PNEUMONIAE WAS USED AS OUTGROUP. THE GENBANK ACCESSION NUMBER IS IN PARENTHESES AFTER THE SPECIES NAME AND ORIGIN OF EACH AGENT. BAYESIAN INFERENCES WERE CARRIED OUT APPLYING THE GTR+I+G MODEL AND 1000 BOOTSTRAP REPLICATES FOR ALL ANALYSES.



SOURCE: The author (2020).

In agreement with the Bayesian inference, the Neighbor-Net network analysis showed a clear phylogenetic separation amongst the hemotropic mycoplasmas found in the white-eared opossums evaluated herein with those previously detected in the North American opossum and other mammals (Figure 8).

FIGURE 8 - NEIGHBOR-NET NETWORK ANALYSIS OF 16S rRNA SEQUENCES (850 BP) OBTAINED FROM OPOSSUM SAMPLED IN THE PRESENT STUDY AND COMPARED TO RELATED HEMOPLASMAS SEQUENCES PREVIOUSLY DEPOSITED IN GENBANK.THE SEQUENCES AMPLIFIED IN THE PRESENT STUDY ARE HIGHLIGHTED IN GREEN.



SOURCE: The author (2020).

The DnaSP analysis based on a fragment of 850 bp of 16S rRNA gene showed that ‘Candidatus Mycoplasma haemodidelphidis’ and the novel hemoplasma species detected in the present study comprised two different genotypes differing by 9 nucleotides. Unfortunately, the lack of 23S rRNA sequences of ‘Candidatus Mycoplasma haemodidelphidis’ available in GenBank database precluded phylogenetic, network and polymorphisms analyses for this molecular marker.

2.4 DISCUSSION

In the present study, 40% white-eared opossums sampled in Canoinhas municipality, SC, southern Brazil was positive for hemoplasmas. The 16S rRNA gene sequences showed that opossums were infected by a potentially novel hemotropic Mycoplasma sp. previously identified in white-eared opossums from two regions of Brazil (MASSINI et al., 2019; GONÇALVES et al., 2020), and closely related to ‘Ca. M. haemodidelphis’.

The phylogenetic analysis of the 16S rRNA gene sequences showed that the hemoplasma detected in white-eared opossums grouped with other hemoplasma sequences previously detected in this opossum species, and clustered separated from 'Ca. Mycoplasma haemodidelphis' detected in North American opossum, supported by a bootstrap of 100% (Figure 6). Corroborating this finding, the Neighbor-Net network analysis evidenced the genetic distinction among the hemoplasma species circulating in opossums from Brazil and the USA.

The identification of a potentially novel hemotropic Mycoplasma sp. infecting white-eared opossums from Brazil is sustained since the detection of this species has been reported in animals inhabiting two different regions and biomes (Cerrado vs Atlantic Forest) of the country (MASSINI et al., 2019; GONÇALVES et al., 2020). Additionally, the areas where *D. virginiana* and *D. albiventris* inhabits do not overlap (Costa et al., 2015; Pérez-Hernandez et al., 2016), making a direct transmission through aggressive interaction (blood during an opossum bite incident) less likely. Previous studies on hemoplasmas have reported that the transmission of bacteria through aggressive interactions may occur between cats (MUSEUX et al., 2009) and wild rodents (*Gerbillus andersoni*) (COHEN et al., 2018). There has not been robust evidence to support the hypotheses that hemotropic mycoplasmas are truly vector-borne pathogens to date, although it is important to point out that white-eared opossums are frequently parasitized by *C. felis* fleas (NASCIMENTO; HORTA, 2014) and *Amblyomma dubitatum* ticks (MASSINI et al., 2019; GONÇALVES et al., 2020), while the North American opossum are mainly infested by *C. felis* fleas and by the American dog tick *Dermacentor variabilis* (DURDEN; WILSON, 1990), for sharing habitats with domestic and other wildlife animals. A previous study has failed to detect hemoplasmas in *A. dubitatum* ticks parasitizing hemotropic mycoplasma-infected white-eared opossums (GONÇALVES et al., 2020), while no studies on hemoplasma detection in *D. variabilis* ticks have been performed to date. Additionally, a previous study has failed in detect hemoplasmas in *Rhipicephalus sanguineus sensu lato* ticks in an animal shelter, suggesting that trans-stadial transmission of canine hemoplasmas does not occur in field conditions (AKTAS; OZUBEK, 2017). Moreover, the potentially novel hemoplasma species detected in white-eared opossums has been found neither in *C. felis* fleas nor cats to date. Further studies should be conducted in order to elucidate the role of vectors in the transmission of this opossum-related hemoplasma species. Accordingly, based on

phylogenetic analysis of the 16S rRNA and 23S rRNA genes associated with epidemiological data, the name ‘Candidatus Mycoplasma haemoalbiventris’ is proposed for this novel organism, which should be further fully characterized by Whole Genome Sequencing approaches.

Previous studies have found hemoplasma prevalence rates of 32.5% (GONÇALVES et al., 2020) and 87.5% (MASSINI et al., 2019) in white-eared opossums from Campo Grande City, Mato Grosso do Sul State, and Maringá City, Paraná State, Brazil, respectively. Herein, 40% opossums tested positive for hemoplasma. Trap area ($p = 0.0887$), gender ($p = 0.7759$) and presence of fleas ($p = 0.3811$) were not associated with hemoplasma infection. Differences in the hemotropic mycoplasma prevalence may be attributed to climate (subtropical vs tropical vs temperate), habitat (undisturbed vs disturbed sites), and sensitivity of the PCR assays.

2.5 CONCLUSION

A potentially novel hemoplasma species is highly prevalent in white-eared opossums from Canoinhas municipality, Santa Catarina State, southern Brazil. Based on phylogenetic analysis of the 16S rRNA and 23S rRNA genes along with epidemiological data, the name ‘Candidatus Mycoplasma haemoalbiventris’ is proposed for this novel organism.

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3 'MOLECULAR DETECTION OF TRYPANOSOMATIDS IN WHITE-EARED OPOSSUMS (*Didelphis albiventris*) FROM SANTA CATARINA STATE, BRAZIL

Abstract

Leishmaniasis and Chagas Disease are neglected zoonoses in public health. Opossums are synanthropic animals and can participate in the zoonosis transmission cycle. Accordingly, the aims of this study were to determine the occurrence and molecularly characterize the trypanosomatids infecting free-ranging opossums and to determine factors associated with these infection in opossums from Canoinhas municipality, Santa Catarina State, southern Brazil. For this purpose, 50 white-eared opossums (33 captured and 17 road-killed animals) were evaluated by PCR assay. A total of 05/50 (10%; 95% CI: 3.33-21.81%) opossums tested positive for trypanosomatids by the PCR assay targeting the *hsp70* gene and ITS1 region. No significant associations were found between gender (p = >0.9999), trap area (p = 0.6199) or sample (p = 0.8807) and positivity to trypanosomatids. In the analysis by BLASTn, the *hsp*70 gene fragments, the samples showed 92.96% - 98.52% identity with *Trypanosoma cruzi* and the ITS1 region 94.03% identity with *Leishmania infantum*, results corroborated by phylogeny. The detection of DNA of etiological agents of Chagas Disease and Visceral Leishmaniasis in opossums, considered wild reservoirs, in the municipality of Canoinhas, Santa Catarina, southern Brazil, is of epidemiological importance due to the absence of human and animal cases reported in the region. The search for vector-borne pathogens with zoonotic potential in wild animals is important to characterize potential reservoirs in the epidemiology of diseases of public health importance.

Keywords: *Trypanosoma cruzi, Leishmania infantum,* Chagas Disease, *Didelphis*, Leishmaniasis.

3.1 INTRODUCTION

The order Kinetoplastida and family Trypanosomatidae include the genera *Trypanosoma* and *Leishmania* and have neglected species of human and animal disease agents. *Trypanossoma cruzi* is the etiological agent of Chagas Disease

(CD). The protozoa infect humans, wild and domestic mammals in endemic areas in Latin American countries. Transmission can occurs by transfusion of infected blood, organ transplantation, the ingestion of contaminated food, congenital, and contact with faeces of vector, the triatomine bugs (BARRETT et al., 2003; WHO, 2013). Visceral Leishmaniasis (VL) is a chronic and systemic disease, which, when left untreated, can progress to death in more than 90% of cases. Caused by the protozoa of the genus *Leishmania*, in the Americas, *Leishmania (Leishmania) infantum* is the species commonly involved in vector transmission (BRASIL, 2019).

Didelphis albiventris (Didelphimorphia) are marsupials, opportunistic, omnivorous, characterized as a highly synanthropic species, due to their ability to adapt to urban and devasted areas (MALTA; LUPPI, 2007; DROZINO et al., 2019). *Didelphis albiventris* is one of the main reservoirs of *T. cruzi* (JANSEN; XAVIER; ROQUE, 2018; DROZINO et al., 2019) and *L. infantum* (CABRERA et al., 2003, BRASIL, 2019).

Visceral Leishmaniasis is endemic in Brazil, occurring in frequent outbreaks, distributed in 21 states, in five Brazilian regions (BRASIL, 2019). In Brazil, in the last 15 years the occurrence of cases of Chagas disease are related to oral transmission through ingestion of contaminated food, mainly in the Amazon region, and vector transmission extra-domestic, with exposure to the wild cycle of the etiological agent (BRASIL, 2021). The state of Santa Catarina is considered non-endemic for vectorial transmission of Chagas disease, with no autochthonous cases and no domiciled triatomines, found only in the forest area. The Canoinhas municipality is considered a non-endemic region for the transmission of Leishmaniasis and Chagas disease. (SANTA CATARINA, 2016; 2020, 2021), but it still has a lot of forest area and abundant wildlife being a favorable environment for the maintenance of zoonotic agents and possible spillover

Accordingly, aims of this study were: i) to determine the prevalence and molecularly characterize the trypanosomatids infecting free-ranging opossums; ii) to determine factors associated with trypanosomatids infection in opossums from Canoinhas municipality, Santa Catarina State, southern Brazil.

3.2 MATERIAL AND METHODS

3.2.1 Study area

The study was carried out in Canoinhas municipality (50° 23' 25" W, 26° 10' 38" S). Canoinhas is located in the northern region of Santa Catarina State, southern Brazil, which is characterized by semideciduous Atlantic Forest fragments and has a temperate climate with an annual average temperature of 17°C.

3.2.2 Ethical approval

This study was approved by the Ethics Committee on Animal Use of the Universidade do Contestado (CEUA-UnC, protocol number 06/18). Animal and laboratory procedures were approved and performed under regulations of the Chico Mendes Institute for Biodiversity Conservation (ICMBio, protocol number 64418-1).

3.2.3 Sampling

Between October 2018 and May 2019, 50 white-eared opossums (29 were females and 21 males) (LEMOS; CERQUEIRA, 2002) were captured in rural and urban areas of Canoinhas municipality, using Tomahawk traps (Figure 1) baited with fruit (HUMBERG et al., 2012; PEREIRA et al., 2018). Sampling was performed by spontaneous demand of the Environmental Military Police of Canoinhas municipality and based on the report of the occurrence of opossums in human dwellings. An effort of 589 trap-night (number of trap * number of days) yielded 33 captures, with a success of 6.92% in rural area (20/289) and 4.33% in urban area (13/300) (MONTEIRO-FILHO; ABE, 1999; VOLOKHOV et al., 2017). Additionally, 17 road-killed opossums were also evaluated (Figure 2). Geographical coordinates from the location of the sampled opossums were recorded (GPSMAP® 64 series, Garmin® International Inc., KS, USA).

After chemical restraint (NASCIMENTO; HORTA, 2014), opossums were identified with ear tagging (CÁCERES; GRAIPEL; CHEREM, 2012) and inspected for ticks and fleas (Figure 3). Subsequently, EDTA blood samples (Figure 4) were taken and stored at -20 °C until PCR analysis. After the procedures, opossums were

monitored and later released at the same place where they were captured. Fragments of spleen (n = 15) and liver (n = 2) tissues were collected from road-killed opossums and stored –20 °C until molecular analysis. The identification of fleas followed Linardi and Guimarães (2000).

3.2.4 DNA extraction

DNA was extracted from all blood and tissue samples using a commercially kit (DNeasy® Blood & Tissue, Qiagen, Germany), according to the instructions on the semi-automated DNA extraction platform Qiacube (Qiagen®). In order to monitor for cross-contamination, ultrapure water was used in parallel as a negative control. DNA was evaluated by fluorimetry for concentration and purity using a Qubit® dsDNA HS Assay (Qubit® 2.0 Fluorometer, Invitrogen, CA, USA).

3.2.5 PCR assays

A PCR for the mammal endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (BIRKENHEUER et al., 2003) was performed in all samples, to ensure successful DNA extraction.

After extraction, samples were submitted to PCR assays targeting the region of ribosomal internal transcribed spacer 1 (ITS1) 300 pb (EL TAI et al., 2000; GRAÇA et al., 2012) and heat shock protein 70 (*hsp70*) gene 234 pb (GRAÇA et al., 2012), according to previously described protocols.

The DNA amplification by PCR was performed in a final volume of 30 µL, containing Tris-HCL buffer (10 mM), $MgCl_2$ (1,5 mM), dNTPs (0,2 mM), Taq DNA Polymerase (2,5 U), DNA of the sample (10 µL) and ultrapure water. The amplification cycles started at 94ºC for 15 min, followed by 35 cycles of 94ºC for 30s, 55ºC for 30s, 72ºC for 30s and a final extension at 72ºC for 10 min. As a positive control for the reaction, 10 ng/µL of the *Leishmania infantum* reference strain (MHOM/BR/74/PP75) was used.*T. cruzi* CL Brener strain.

The PCR products were visualized in 2% agarose gel red and purified using the Kit QIAquick PCR Purification (Qiagen®).

3.2.6 Amplification of kDNA by qPCR to *Leishmania infantum*

After extraction, the samples were amplified in triplicate using hydrolysis probes (TaqMan® system) on the StepOne™ platform (Applied Biosystems®, by Thermo Fischer Scientific®, MA, EUA).

The hydrolysis probe (TaqMan® MGB) and the PCR were designed to target the conserved regions kDNA of the *L. infantum*. The primers LEISH-1 (5'-AACTTTTCTGGTCCTCCGGGTAG-3') e LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3') and hydrolysis probes (FAM- 5'AAAAATGGGTGCAGAAAT- 3'- NFQ -MGB) used were described previously in the protocol of FRANCINO et al. (2006).

The final reaction volume was 25 µL, 5 µL of sample and 20 µL of mix containing 12.5 µL of Universal Mastermix (Applied Biosystems®, by Thermo Fischer Scientific®, MA, USA), 1.5 µL of LEISH-1 and LEISH-2 primers at 900 nM and 2.5 µL of the probe at 200 nM and 2 µL of ultra-pure water (SOLCÀ et al., 2014). The protocol of the cycles was: 1 cycle of 50° C for 2 min, 1 cycle of 95° C for 10 min and 40 cycles of 95° C for 15 seconds and 60° C for 1 min.

The reaction was performed in a 48-well plate (Applied Biosystems®, by Thermo Fischer Scientific®, MA, USA) that was sealed with adhesive film (Applied Biosystems®, by Thermo Fischer Scientific®, MA, USA) after pipetting the reaction.

For quantification of the number of genome equivalents (gEq) of *L. infantum* in the DNA samples assessed in the study by qPCR reaction, a standard curve made with *L. infantum* culture representing the points of 2,500, 250, 25, 2,5, 0,25 was used for each plate (CAMPOS et al., 2017).

In each amplification, positive and negative controls were used and the determined threshold was 0.1 for all samples, by determining the point at which the fluorescence emitted exceeded the limit considered negative. The limit for obtaining the detectable results was set at 37 cycles. Samples that showed a fluorescence signal after cycle 37 were considered undetectable (CAMPOS et al., 2017).

3.2.7 Sequencing

Sequencing was performed at the facilities of Oswaldo Cruz Foundation (IOC) (Genomic Platform - DNA sequencing, PDTIS-FIOCRUZ) with the PCR product at a

concentration of 10ng DNA and primers ITS1 and *hsp70* at 3.2 pmols in the sequencer ABI3730xl (Thermo Fischer Scientific).

3.2.8 Phylogenetic analysis

The consensus sequence was aligned with other sequences retrieved from GenBank using the MAFFT software, version 7 (KATOH; STANDLEY, 2013). The best evolutionary model was chosen using the jModelTest2 software (version 2.1.6) on XSEDE (DARRIBA et al., 2012) via the CIPRES Science Gateway (STÖVER; MÜLER, 2010). The phylogenetic analyses were based on Bayesian inference (BI) method. The BI analyses were performed using MrBayes 3.1.2 (RONQUIST; HUELSENBECK, 2003) via the CIPRES Science Gateway. Markov chain Monte Carlo simulations were run for 10^6 generations with a sampling frequency of every 100 generations and a 25% burn-in. The phylogenetic trees were edited using TreeGraph 2.0.56-381 beta software (STÖVER; MÜLER, 2010).

3.2.9 Statistical analyses

The Fisher's exact test was used to determine the difference between whether individual factors (gender, trap area and collected material) e associated with infection by trypanosomatids. Odds ratio (OR), 95% confidence interval and p values were calculated for each variable with results considered significantly when $p < 0.05$. Data were analyzed in program GraphPad Prism (version 6).

3.3 RESULTS

All samples consistently amplified the mammal endogenous *gapdh* gene. A total of 05/50 (10%; 95% CI: 3.33-21.81%) opossums tested positive for trypanosomatids by the PCR assay targeting the ITS1 region and *hsp70* gene. One opossum was positive for both. A total of 04/50 (8%; 95% CI: 2.22-19.23%) opossums tested positive (blood sample) for trypanosomatids by the PCR assay targeting the *hsp70* gene and a total of 02/50 (4%; 95% CI: 0.49-13.71%) opossums tested positive (one blood sample/ one spleen sample) for trypanosomatids by the PCR assay targeting the ITS1 region. (Table 2).

TABLE 2 - POSITIVE PCR ASSAY TARGETING THE HSP70 GENE AND REGION ITS1 IN OPOSSUMS, CANOINHAS, SANTA CATARINA STATE, SOUTHERN BRAZIL, 2020.

PCR	+/n (%)	CI % (95% CI)
hsp70	4/50 (8)	2.22-19.23
ITS1	2/50 (4)	0.49-13.71
Coinfection	1/50 (2)	0.05-10.65

+, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval.
SOURCE: The author (2020).

No significant associations were found between gender (p = >0.9999), trap area (p = 0.6199) or sample collected (p = 0.8807) and positivity to trypanosomatids (Table 3).

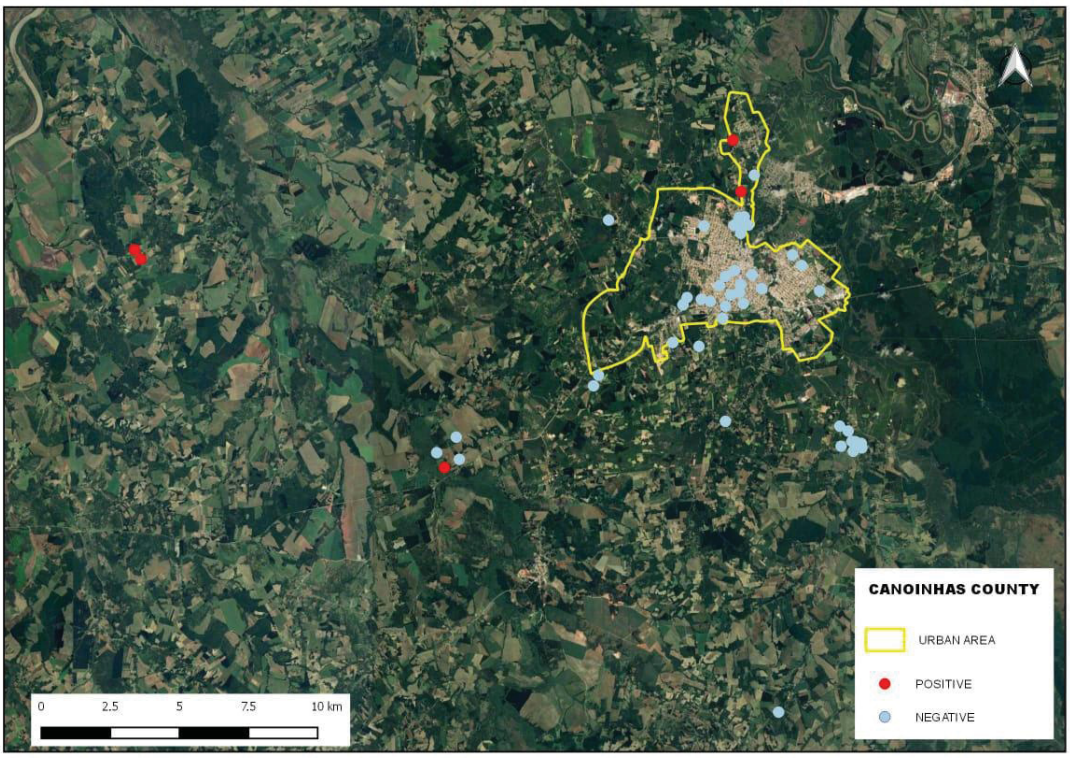
TABLE 3 - OCCURRENCE OF TRYPANOSOMATIDS SPECIES IN OPOSSUMS WITHIN EACH VARIABLE STUDIED, CANOINHAS, SANTA CATARINA STATE, SOUTHERN BRAZIL, 2020.

Variable		+/n	OR (95% CI)	p-value
Gender	Male	2/21 (9.52)	0.9139 (0.1004-6.709)	>0.9999
	Female	3/29 (10.34)		
Trap Area	Rural	3/20 (15.00)	2.4240 (0.3297-22.15)	0.6199
	Urban	2/30 (6.67)		
Sample	Viscera	1/17 (5.88)	0.4595 (0.0173-4.022)	0.8807
	Blood	4/33 (12.12)		

+, Number of positive animals; n, number of analyzed samples; 95% CI, 95% confidence interval.
OR: ODDS ratio
SOURCE: The author (2020).

The geographic distribution of trypanosomatids-PCR-positive and negative opossums is shown on Figure 9.

FIGURE 9 - GEOGRAPHIC POSITION OF POSITIVE AND NEGATIVE OPOSSUMS (n=50) FOR TRYPANOSOMATIDS.



SOURCE: The author (2020).

The sequencing of *hsp70* gene fragments was similar to that of *Trypanosoma cruzi*. The sequencing of fragments from the ITS1 region showed similarity to *Leishmania infantum*, these samples were positive for *Leishmania infantum* in qPCR (Table 4).

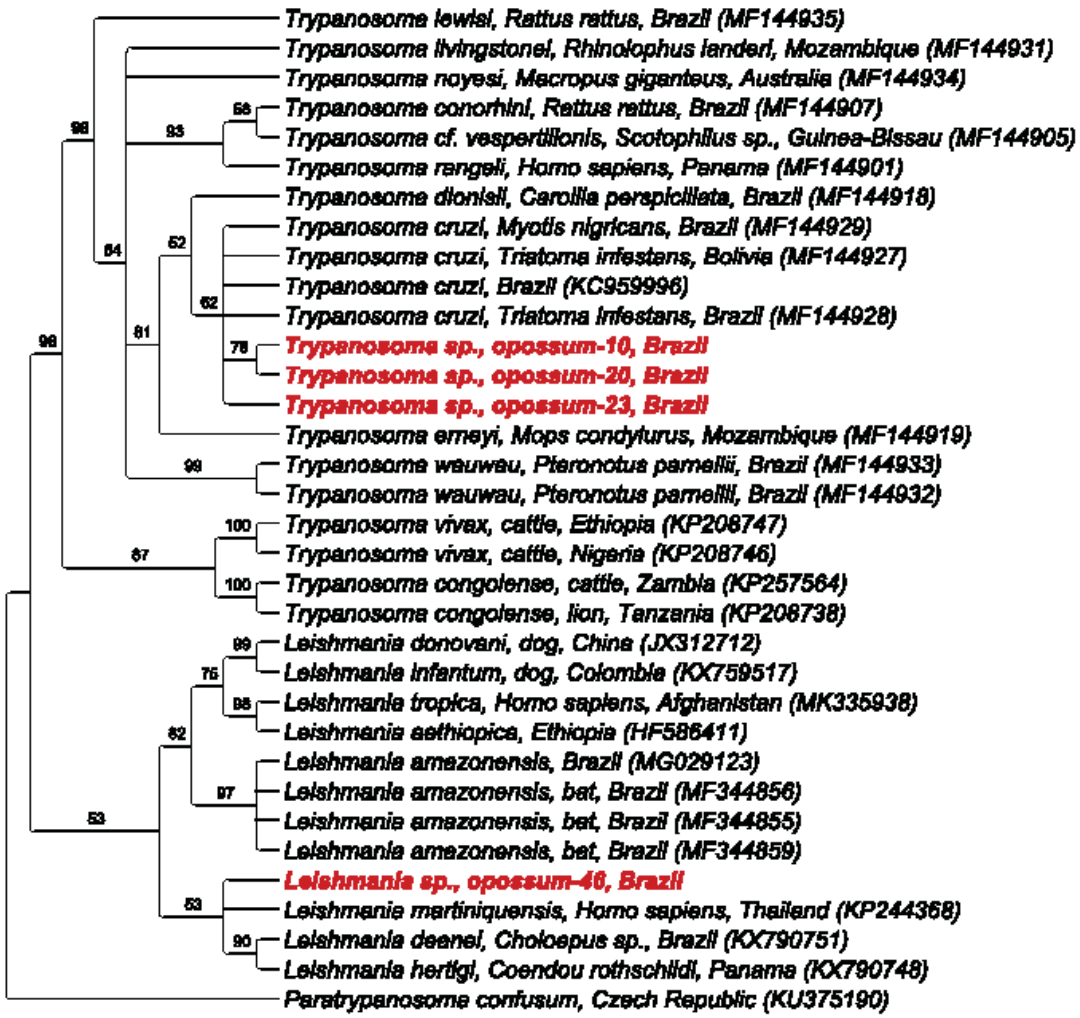
TABLE 4 - SEQUENCING OF FRAGMENTS PCR POSITIVE PCR ASSAY TARGETING THE HSP70 GENE AND REGION ITS1 IN OPOSSUMS, CANOINHAS, SANTA CATARINA STATE, SOUTHERN BRAZIL, 2020.

PCR positive	***qPCR***	***Opossum***	***Percent identify (%)***	***Description***	***GenBank® accession***
hsp70	-	10	98.52	*Trypanosoma cruzi*	KC959996.1
hsp70	-	20	92.96	*Trypanosoma cruzi*	KC959996.1
hsp70	-	23	94,53	*Trypanosoma cruzi*	KC959996.1
hsp70	-	46	90.05	*Leishmania sp.*	MF344859.1
ITS1	positive	02	94.03	*Leishmania infantum*	MF688836.1
ITS1	positive	20	-	*Leishmania infantum*	-

qPCR: Efficiency: 83.27%, R^2: 0.942, *y*-intercepto: 35.21
SOURCE: The author (2020).

The figure 10 showed phylogenetic tree based on partial sequences of the *hsp70* gene. The results obtained were corroborated by the phylogeny, showed figures 10 and 11.

FIGURE 10 - PHYLOGENETIC TREE BASED ON PARTIAL SEQUENCES OF THE *hsp70* GENE, SHOWING THE RELATIONSHIP BETWEEN THE TRYPANOSOMATIDS DETECTED IN THE WHITE-EARED OPOSSUMS (*Didelphis albiventris*) sampled in THIS STUDY AND OTHER TRYPANOSOMATIDS.

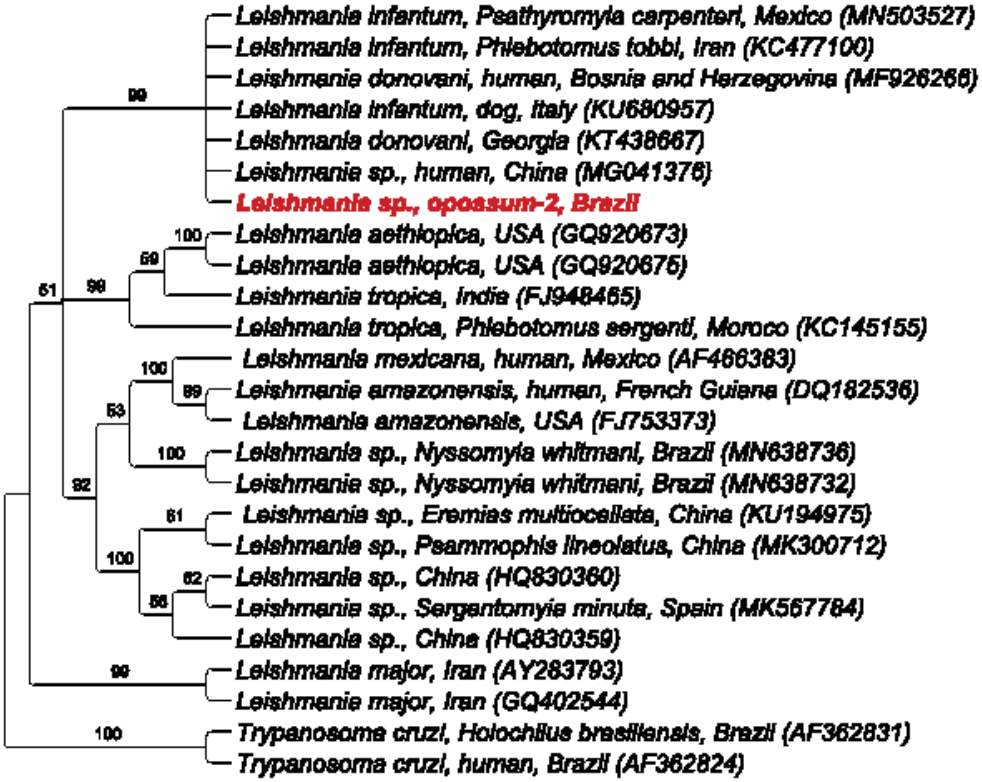


SOURCE: The author (2020).

SUBTITLE: The genbank accession number is in parentheses after the species name and origin of each agent. Based on Bayesian inference method. *Paratrypanosoma confusum* was used as outgroup.

The figure 11 showed phylogenetic tree based on partial sequences of the ITS1 gene.

FIGURE 11- PHYLOGENETIC TREE BASED ON PARTIAL SEQUENCES OF THE ITS1 REGION , SHOWING THE RELATIONSHIP BETWEEN THE TRYPANOSOMATIDS DETECTED IN THE WHITE-EARED OPOSSUMS (*Didelphis albiventris*) sampled in THIS STUDY AND OTHER TRYPANOSOMATIDS.

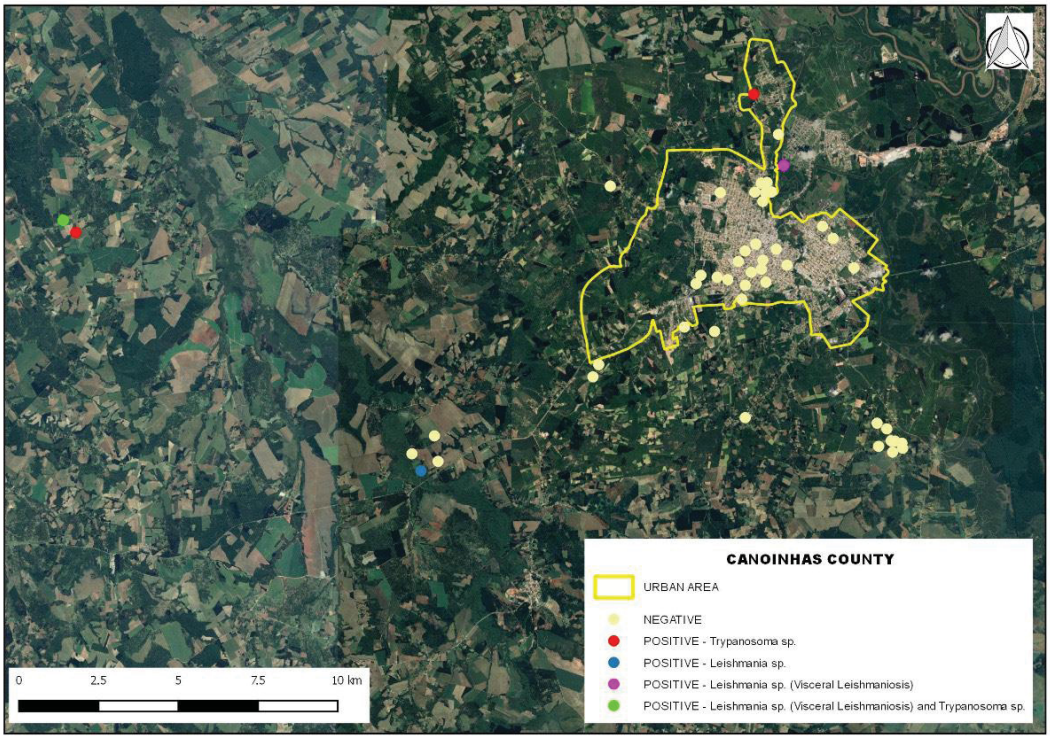


SOURCE: The author (2020).

SUBTITLE: The genbank accession number is in parentheses after the species name and origin of each agent. Based on Bayesian inference method. *Trypanosoma cruzi* was used as outgroup.

The Figure 12 presents the geographic distribution according to the identity of the agent obtained in the sequencing of the trypanosomatid-PCR-positive and negative samples.

FIGURE 12 - GEOGRAPHIC POSITION OF CAPTURED POSITIVE AND NEGATIVE OPOSSUMS ACCORDING TO THE IDENTITY OF THE AGENT OBTAINED IN THE SEQUENCING.



SOURCE: The author (2020).

3.4 DISCUSSION

The detection of DNA of causal agents of Visceral Leishmaniasis (*L. infantum*) and Chagas Disease (*T. cruzi*) in opossums, in the municipality of Canoinhas is an important epidemiological data, since opossums are considered wild reservoirs of the studied pathogens and the municipality is considered a non-endemic region for the diseases studied and there are no studies reporting phlebotomine and triatomine vectors in the area covered by the study to date.

Domestic dogs and several wild mammal species, especially carnivores, rodents, and marsupials, have already been identified as potential reservoir hosts of *L. infantum* and *T. cruzi* in Brazil, but no previous studies have identified natural infection in a domestic or wild vertebrate host in Canoinhas. Here, we present evidence of a possible sylvatic enzootic transmission cycle of trypanosomatideos in *Didelphis albiventris* hosts in the Municipality of Canoinhas, in the north region of the State of Santa Catarina. The risk of vector transmission depends on the existence of native triatomine species; the presence of mammals reserving *T. cruzi* close to

human populations (BRASIL, 2021). Currently, the main form of transmission of CD occurs through the ingestion of edible contaminated by metacyclic forms of *T. cruzi.* (JANSEN; XAVIER; ROQUE, 2018). Santa Catarina is non-endemic area for the traditional vectorial transmission of CD because the triatomines are limited to wild areas, with no autochthonous cases of the disease by vectorial transmission. One outbreak of the disease were reported in 2005 with 23 people infected by the oral-transmission of the parasite, by ingestion of sugar cane juice (DIAS, 2006; SANTA CATARINA, 2021).

Studies conducted in the five biomes of Brazil, between 1992 and 2017, showed that 17% wild mammals were seropositive (by indirect immunofluorescence test) of *T. cruzi* and 8% of all animals displayed positive hemocultures. Of the sampled *Didelphis* 22% displayed positive hemocultures and 36% were seropositive. In the Atlantic Forest biome to determine the transmission cycle of the *T. cruzi* wild environment, *Didelphis* sp. 18% displayed positive hemocultures and 27% were seropositive (JANSEN; XAVIER; ROQUE, 2018).

Opossum can be infected orally preying on small mammals or triatomines; in addition, *T. cruzi* can multiply in the scent glands, adjacent to de cloaca, as epimastigota forms and for its differentiation to infective metacyclic trypomastigote form, stages that correspond to the cycle of *Trypanosoma cruzi* in the intestinal lumen of its insect vector (DEANE; LENZI; JANSEN, 1984).

The human oral transmission can occur when food is accidentally contaminated with the parasite, triatomine or its feces; through the ingestion of raw or undercooked game meat or food contaminated by the secretion of the anal glands of infected marsupials (DIAS, 2006; SANTA CATARINA, 2021; BRASIL, 2019; BRASIL, 2021). Study conducted in riverine communities an Amazon floodplain region, demonstrated hunting activity and use opossum of food and medicinal. *Didelphis marsupialis*, is used in the treatment of diseases, such as rheumatism, asthma, sore throat and inflammation (BARROS; AZEVEDO, 2014). Considering the participation of opossum in the wild cycle of protozoa, the cultural habit in vulnerable populations is an aggravating factor in public health measures.

Previous study for the detection of *Leishmania* sp DNA in opossums showed 16% (4/25) of positive animals in Alagoas and Pernambuco for the *L. (V.) braziliensis* complex (SILVA et al., 2016). In Brasilia, an epidemiological study showed the seroprevalence of 33.28% (233/700) of *Leishmania* sp. in humans, among the factors

associated with positivity, the presence of opossums close to homes were reported (CARRANZA-TAMAYO; WERNEK, ROMERO, 2016). In Minas Gerais state, a serological study in opossums detected 21.6% (24/111) animals positive by indirect fluorescence antibody test (IFAT) and 20% (5/20) samples analyzed with PCR tested positive for the presence of *Leishmania*-specific DNA, the study highlighted the potential of opossums as wild reservoirs in Brazil (SCHALLIG et al., 2007).

Phlebotomines are collected mainly in rural areas, in dwellings with precarious conditions of hygiene, accumulation of organic matter, and facilities near remaining forests. In Santa Catarina, the potential vectors involved in maintaining LV transmission between dogs are species the genus *Lutzomia.* In Florianópolis, the phlebotomes have been identified: *Nissomyia neivai; Migoneimyia migonei; Pintomyia fischeri*. The municipality presents fragments of the Atlantic Forest biome favorable to the proliferation of phlebotomes, native to this environment, and which supposedly have been maintaining the transmission of the disease among dogs. The municipality of Canoinhas does not present entomological information (SANTA CATARINA, 2020).

Although males demonstrate nomadic behavior with the greatest potential for exposure to infectious agents no significant associations were found between gender and positivity to trypanosomatids. *Didelphis* are nocturnal, nomadic (male), agile climber, with high levels of parasitaemia with infectivity (JANSEN; XAVIER; ROQUE, 2018). Regarding the area (rural / urban), when observing the geographic distribution of positive animals (Figure 9), there is a predominance of positive animals in the rural area and peri-urban area but no significant associations were found between trap area and positivity to trypanosomatids.

3.5 CONCLUSION

The detection of trypanosomatid DNA, etiological agents of Chagas disease and visceral leishmaniasis in opossums, is of epidemiological importance because absence of cases in the non-endemic region. Opossum participate in the wild enzootic cycle of diseases in the studied area. Future studies for vector detection and reservoir capacity of opossums will be important to monitor the epidemiology of diseases. However, the human population in the studied area is at risk of exposure to agents considering the synanthropic behavior of opossums.

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4 CONSIDERAÇÕES FINAIS

Tripanosomatídeos e hemoplasmas estão presentes em gambás de vida livre do município de Canoinhas, Santa Catarina, sul do Brasil. O sequenciamento e a análise filogenética dos fragmentos do gene 16S e 23S rRNA confirmaram que os animais foram infectados por um potencial novo *Mycoplasma* sp hemotrópico relatado anteriormente em gambás-de-orelha-branca do Brasil. O nome *"Candidatus* Mycoplasma haemoalbiventris" foi proposto para este novo organismo.

Não foi encontrada associação significativa entre as variáveis estudadas e animais positivos para tripanosomatídeos (sexo, área e amostra) e hemoplasma (sexo, área e infestação por pulgas). No sequenciamento dos fragmentos do gene *hsp70* e região ITS1, as amostras apresentaram 92,96% - 98,52% de identidade com *Trypanosoma cruzi*, e 94,03% de identidade com *Leishmania infantum*, resultados corroborados pela filogenia.

A detecção de DNA de agentes etiológicos tripanosomatídeos da Doença de Chagas e Leishmaniose Visceral em gambás, considerados reservatórios silvestres, é de importância epidemiológica pela ausência de casos humanos na região.

Gambás participam do ciclo enzoótico das doenças na área do estudo. Pesquisas futuras objetivando a vigilância epidemiológica de vetores e para caracterizar a relação parasita-hospedeiro em gambás como reservatórios amplificadores e mantenedores dos agentes são importantes para monitoramento epidemiológico das doenças. A população humana, na área de estudo, apresenta risco de exposição aos agentes considerando o comportamento sinantrópico dos gambás.

ANEXO 1 – CERTIFICADO DA COMISSÃO DE ÉTICA NO USO E EXPERIMENTAÇÃO DE ANIMAIS - CEUA

Universidade do Contestado – UnC
Comissão de Ética no Uso e Experimentação de Animais - CEUA

CERTIFICADO

Certificamos que a proposta intitulada **"Patógenos transmitidos por vetores em gambás do município de Canoinhas no estado de Santa Catarina"**, registrada com o **nº 06/18**, sob a responsabilidade de **Giane Helenita Pontarolo** - que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade do Contestado, em 26/09/2018.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	Até fevereiro de 2021
Nº da Solicitação ou Autorização SISBIO	64418-1
Atividade(s)	(X) Captura ( ) Coleta de espécimes (X) Marcação (X) Outras – coleta de amostras de sangue e ectoparasitas
Espécies/Grupos Taxonômicos	Ordem Didelphimorphia, família Didelphidae, subfamília Didelphinae, gênero *Didelphis*
Local(is) de realização das atividades	Animais de vida livre capturados na área urbana e/ou rural do Município de Canoinhas SC

Mafra, 28 de setembro de 2018.

Profª. Dra. Daniela Pedrassani
Coordenadora CEUA/UnC

ANEXO 2 – AUTORIZAÇÃO DO INSTITUTO CHICO MENDE DE CONSERVAÇÃO DA BIODIVERSIDADE

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 64418-1	Data da Emissão: 25/09/2018 16:51:50	Data da Revalidação*: 25/09/2019
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Giane Helenita Pontarolo	CPF: 042.691.509-71
Nome da Instituição: Universidade Federal do Paraná	CNPJ: 75.095.679/0001-49

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Pesquisa de patógenos nas amostras biológicas por PCR e/ou sorologia	09/2018	02/2021
2	Caracterização molecular dos patógenos identificados	09/2018	02/2021
3	Processamento, identificação e armazenamento das amostras biológicas	09/2018	02/2021
4	Captura, transporte e marcação de gambás (Didelphis)	09/2018	02/2021
5	Coleta e transporte de amostras biológicas (ectoparasitas, sangue, soro, tecidos e carcaças)	09/2018	02/2021

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Rafael Sachet Rodrigues	Colaborador	699.424.390-72	Brasileira
2	Juliano Bioichi	Colaborador	098.961.529-42	Brasileira
3	Rubiana Carvalho dos Santos	Colaboradora	069.114.799-08	Brasileira
4	Daniela Pedrassani	Colaboradora	808.175.729-53	Brasileira
5	Ivan Roque de Barros Filho	Pesquisador	093.151.948-96	Brasileira
6	RAFAEL F C VIEIRA	Pesquisador	041.694.404-39	Brasileira
7	Fabiano Borges Figueiredo	Pesquisador	283.115.838-97	Brasileira
8	djalmo gervasio rodrigues	Colaborador	085.913.439-30	Brasileira

Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontre-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional de Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando de violação de legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
3	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
4	O titular de licença ou autorização e os membros de sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.

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